Nanotechnology-based approaches for the treatment of migraine using intranasal route

SUMMARY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (Ph.D.)

IN

PHARMACEUTICAL SCIENCES

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1. BACKGROUND OF THE STUDY

A neurological condition, migraine is also known as “cluster headache” causes throbbing and pulsating pain in head due to dilation of intracranial nerves. [1,2] Typical symptoms include nausea, vomiting, and increased sensitivity to strong light, loud noises, and odors. Aura is another neurological symptom connected to migraine (the perception of strange light or smell on onset of attack). If left untreated, this strong headache could linger anywhere from few hours to a few days. [3] The third most common disease rated in the world is migraine which is more prevalent than diabetes, epilepsy and asthma combined together. According to statistics by gender, children (10%), men (6%) and women (18%) suffer with migraines, which are most common in people between the ages of 20 and 50 (Figure 1). [4] Women are three times more impacted than males due to a variety of factors, including hormonal changes during puberty, pregnancy, menopausal stage, and mental disorders.

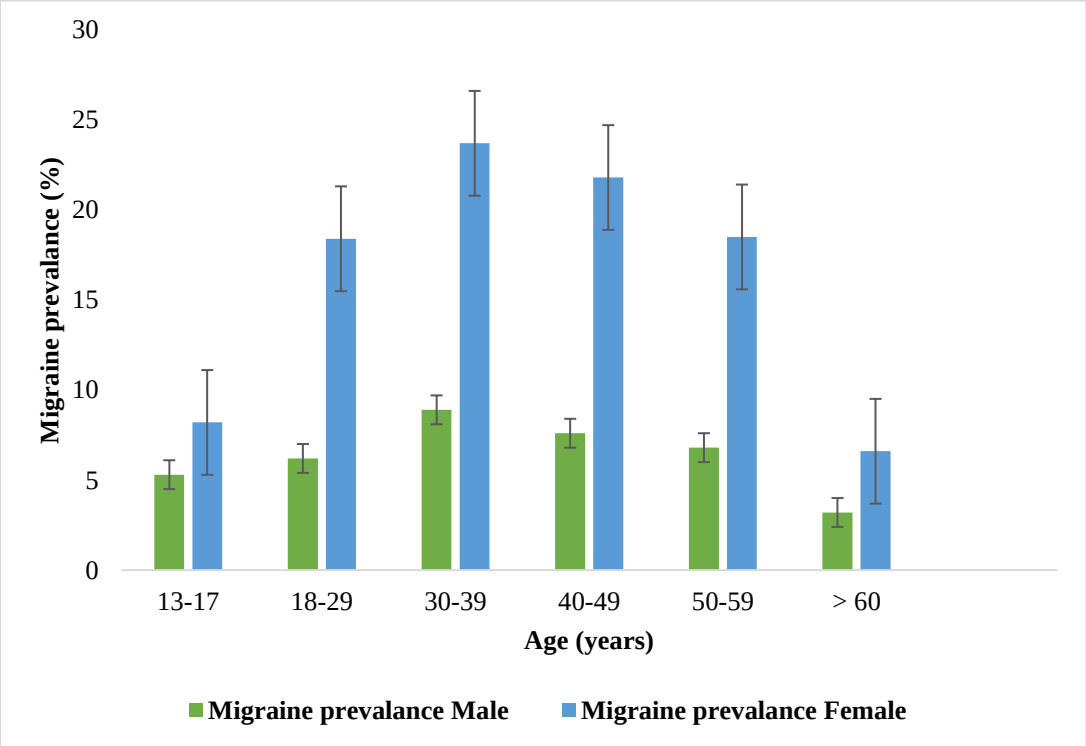

Figure 1. Population statistics of migraine patients (2020) in India

There are four phases of migraine attack: Prodrome stage lasting for 48 h before an attack, aura stage lasts for an hour preceding the headache, the main stage headache that lasts for about 4 to 72 h and post-drome stage wherein the pain gradually disappears.

The epidemiology of migraine has identified many risk factors for its prevalence and disease progression is associated with two factors like biological and psychological factors. Among biological factors, the main is hormonal imbalance, [5] where hormones control the chemicals in the brain that are associated with pain sensation and hence any hormonal imbalance or fluctuations trigger migraine. Metabolic factors include group of metabolic disorders like diabetes, obesity, hypertension and dyslipidaemia related to the sedentary urban lifestyle.[6] Psychological factors include stress as the most important contributing factor in the prevalence of migraine. Other factors acting as triggering agents for migraine are anxiety disorders, phobia and panic disorders.

2. RATIONALE

Ergotamine shows short half-life (2 h) but its biological action (vasoconstriction effect) lasts for about 24 h. This is because the active metabolite is in circulation for prolonged period. Ergotamine drug has two phase elimination kinetics - first phase lasting for 90 min and second phase for 20 h. The oral route shows less bioavailability (12%) and hence alternate routes like parenteral, inhalation and rectal routes for its effective delivery are required for further research.

The injection, inhalation and rectal routes showed variable bioavailability from 14% -60% compared to oral route 12%.[7] However, nasal route seems more promising because of its rapid absorption, high vasculature, nose-to-brain delivery and low concentrations. The plasma levels are reached within 5 min post-inhalation for ergotamine. The marketed preparations available for effective treatment of migraine include orally disintegrating tablets (ODT), sublingual tablets, nasal drops, nasal sprays and vaccines. Oral dosage forms like tablet

formulations (orally disintegrating tablets and sublingual tablets) administration possess lot of drawbacks like extensive first-pass metabolism of drugs. Also, oral dosage forms exhibit erratic absorption in the GIT. Further, inadequate therapeutic dose is achieved after oral administration as migraine is associated with nausea and vomiting. This led to development of injectable dosage form to provide the rapid onset of action. However, self-administration with patients and pain associated during injectables or hospitalisation requirements lead to reduce patient compliance and higher cost of treatment. An alternative non-invasive route viz. intranasal route is the most promising effective route for drug delivery to the brain without crossing the BBB. Intranasal administration delivers medication through various pathways like olfactory, trigeminal, and vascular transport directly to the brain without crossing the BBB.[8] The drug of choice in migraine treatment is ERT because most formulations of ERT combined with CNS stimulant caffeine (CAF) as CAF worked synergistically with ERT to relieve the migraine symptoms by forming soluble molecular complex that increase the ERT solubility with significant rate and extent of absorption of ERT. [9,10] Though the triptans proved to be a better choice than ERT, they exhibited low elimination half-life 2-4 h. ERT shows longer duration of action as compared to triptans and is equally potent and safe.

3. OBJECTIVES

The objectives of present research work were:

- To stabilise ergotamine formulation for effective intranasal delivery to the brain in the treatment of migraine using nanotechnology approaches as
    - Polymer nanoparticles (PNP) viz., nanosponges and nanospheres in microneedles
    - Lipid nanoparticles (LNP) called vesosomes
    - Lipid polymer hybrid nanoparticles (HNP)

With permeation enhancers like caffeine, if required

- Formulation and characterisation of nanosponges
- Formulation and characterisation of nanospheres in microneedles
- Formulation and characterisation of vesosomes
- Formulation and characterisation of hybrid nanoparticles
- Evaluation of all nanoparticulate formulations by conducting *in-vivo* studies

Characterisation of nanosponges, vesosomes, hybrid nanoparticles and microneedles

The optimized nanoparticulate formulations (nanosponges, nanospheres in microneedles, vesosomes and hybrid nanoparticles) were designed for intranasal drug delivery from nose-to-brain without crossing the blood-brain barrier and effectively target the brain to offer controlled release rate that result in reduction of dosing frequency with wider patient acceptance. All the formulations used fixed-dose combination of ERT with CAF for synergistic action to improve the solubility and nano-encapsulation technique impart better stability to the formulation. These formulations were characterised for following parameters:

- Morphology characteristics (Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM))
- Particle size, zeta potential and polydispersity index
- Solubility studies
- % Entrapment efficiency
- *In-vitro* release studies
- *Ex-vivo* release studies
- Structural chemistry and interactions (Fourier transform infrared (FTIR) spectroscopy, Differential scanning calorimeter (DSC), NMR spectroscopy and X-ray diffraction (XRD) spectroscopy)

- Stability studies
- Animal studies (Pharmacokinetics studies, Pharmacodynamics studies, Histopathology studies and Serotonin toxicity studies)

4. APPROACHES TO THE ISSUES

Nanotechnology offers various advantages owing to its nano size like higher surface area-to-volume ratio, improved permeability and better muco-adhesion.[11] The surface properties are increasingly becoming an intrinsic parameter of a particle's or material's possible activities at the nanoscale due to the surface-to-volume ratio. Ease of fabrication technique is another advantage of this technology.[12] Nanotechnology involves nanocarriers for targeted drug delivery like polymeric nanoparticles, lipid nanoparticles, lipid-polymer hybrid nanoparticles and microneedles.

Polymeric nanoparticles in the form of nanosponges are colloidal structures made up of tiny microscopic particles with pores that can encapsulate various molecules like volatile oils, anticancer drugs, proteins and peptides, hydrophilic and lipophilic molecules thus enhancing the solubility of poorly soluble molecules.[13] Nanosponges act as three-dimensional network of long chain polyesters constituting a backbone and depicting the size of a virus. These nanosponges are porous, non-toxic, insoluble in water and organic solvents with stability at wider pH range of 1 -11 and up to higher temperatures of 300°C. Though lipophilic, they are easily dispersible in water and possess the ability to mask unpleasant flavours and convert liquids to solids post entrapment in their network. [14] The network constituting of polyesters like polyglycolic acid and cyclodextrin that allow for higher payload and controlled release pattern by attaching on the target sites within the body. The release from nanosponges can be altered by varying the ratio of polymer to crosslinker.[15]

Nanospheres in microneedles (MNs) offer an interesting drug transport nanocarrier that combine the advantages of non-invasive transdermal delivery of drugs along with invasive drug

delivery using conventional hypodermic needles. [28] MNs are micro-dimensional needles similar to conventional hypodermic needles arranged in form of array for various interdisciplinary fields to deliver hydrophilic and lipophilic drugs including a diversified range of small and large molecules, proteins, peptides and vaccines. MNs unlike a transdermal patch penetrate skin by creating microchannels in the stratum corneum which is main blockade for transdermal delivery and act with negligible pain and penetration depth of 80-200 nm without disruption of dermal layer that leads to skin permeability enhancement and effective delivery of drugs at the target sites. [29,30]

Lipid nanoparticles viz., vesosomes are the nested bilayer compartments of lipids with various inner vesicles segregated by an exterior membrane and each compartment encapsulates diverse materials with varied bilayer compositions like phosphatidylcholines, phosphatidylglycerols and phosphatidylserines. [16][17] These are mainly multivesicular vesicles (MVVs) or liposome-in-liposome structures formed due to interdigitation of saturated phospholipids in presence of alcohols or glycols. [18] Lipid nanoparticles exhibit superior biocompatibility owing to structural similarity to biological membranes, ease of manufacturing and favourable safety profile but face the drawbacks of lacking structural integrity due to drug leakages and instability during storage.

The drawbacks of both polymeric nanoparticles and liposomes can be overcome by the new technique lipid polymer hybrid nanoparticles (LPHNP). These are core shell type structures that possess synergistic properties of both liposomes and polymeric nanoparticles. [19,20] The HNP structure constitutes three functional components, an inner core of polymer containing therapeutic drug (BCS class II and IV) with high payload, surrounded by a layer of lipids around the core of polymer that offers biocompatibility and augments drug retention within the polymer core. Moreover, the third layer of lipid PEG enhances stability and prolongs *in-vivo* circulation times by steric stabilization. [21,22,23] The polymer core and surrounding lipid

layers are linked via van der Waals forces of attraction or hydrophobic interactions whereas the water-soluble polymer core is coupled via covalent bonds with the surrounding lipids in hybrid nanoparticles. [24,25] Most HNPs are synthesized by the single-step method that comprises simple mixing of lipid aqueous dispersion with polymer organic solution using the nanoprecipitation method. [26,27]

5. MATERIALS AND METHODS

5.1 Materials

Ergotamine Tartrate (ERT) procured from Teva Czech Industries, Czech Republic. Caffeine (CAF) was received as a gift sample from Aarti drugs, Mumbai, India. Hydroxypropyl ß-cyclodextrin (HCD) was obtained from Signet, India whereas Toluene diisocyanate (TDI), Trimethyl amine (TEA), potassium hydroxide (KOH) and dichloromethane (DCM) were purchased from Sigma Aldrich, USA. Lipoid PC 16:0/16:0 (Dipalmitoyl phosphatidylcholine/DPPC) and Lipoid S-100 (Phosphatidylcholine from Soyabean) were procured from Lipoid, Germany. Cholesterol was obtained from Avanti Polar Lipids, Germany. Porcine pancreatic phospholipase enzyme (PLA2) was purchased from Krishgen Biosystems, USA. Resomer® RG 503 H (Poly-acid terminated, lactide: glycolide 50:50, $M_{W:}$24,000-38,000) procured from Evonik, USA. Polyethylene glycol (PEG-2000, M_W:1900-2200) and Polyethylene glycol (PEG-400, MW: 280-420) were acquired from Merck (USA). Poly lactic-co-glycolic acid (PLGA) (lactide/glycolide ratio 75:25) was received from Evonik, India. Polyvinyl alcohol (PVA; Mw: 85,000 -125,000) procured from Spectrum chemicals, USA whereas polyvinylpyrrolidone (PVP K-90; MW: 360,000) from BASF, Germany. Water purified from MilliQ® Integral Water Purification System was used for preparation and analysis. Analytical reagent grade chemicals were used throughout the experiments.

5.2 Methods of preparation

5.2.1 Method of preparation for nanosponges (NS)

The cost-effective and easy to scale-up manufacturing method i.e., kneading method utilized for ERT-HCD inclusion complex and encapsulated via interfacial polymerization technique to yield nanosponges. A statistical approach used to find the best ratio of ERT-HCD inclusion complex with caffeine wherein 3^2 Full Factorial design employed in the experiments using the Design-Expert software. The HCD (dehydrated by drying in an oven at 100ºC for 2 h) was triturated with ERT varying the molar ratios (1:3) of ERT: HCD using ethanol as a solvent which was then dried at ambient temperature. The aqueous suspension of inclusion complex in KOH was added dropwise to organic solution and stirred for 30 min using magnetic stirrer (Remi, India) at 450 rpm. The resulting suspension filtered through Whatman filter paper under vacuum and the precipitate washed further with double distilled water to remove the undesirable residue and free drug, if any. [31]

5.2.2 Method of preparation for nanospheres in microneedles

The nanospheres obtained by employing multiple emulsion approach i.e., water-in-oil-in-water (w/o/w) using solvent-diffusion-evaporation process. Initially primary emulsion (w/o) formed that involved mixing two phases: organic phase and aqueous phase wherein organic phase comprised of PLGA and ERT dissolved in DCM whereas aqueous phase constituted of PVA, sodium bicarbonate and CAF dissolved in distilled water. Microneedles were prepared using a polymer matrix of PVA and Povidone K-90 and casting the polymer blend loaded with PLGA nanospheres in sterile silicon molds. PLGA nanospheres equivalent to 1 mg ERT were mixed with the polymer matrix and sonicated for 30 min to remove the air entrapped during stirring method. This viscous dispersion was poured carefully to avoid air entrapment in pre-sterilized silicon molds with an area of 2 x 2 cm^2 and dried in an oven at 50ºC for 45 min. Post drying the MN array was removed, covered with aluminium box and kept in a desiccator under vacuum till further usage.[30]

5.2.3 Method of preparation for vesosomes (VS)

Thin film hydration method employed for small unilamellar liposome formation. Lipoid S 100 and cholesterol were mixed in a ratio of 2:1 and then dissolved in ethanol and chloroform mixture. ERT was added to lipid solution and then sonicated for 3 min to dissolve completely in lipids. This mixture in round bottom flask (RBF) was loaded on to Rota-evaporator and thin film formed was hydrated with phosphate buffer pH 6 and further micro-fluidized at 15,000 psi and 5 cycles for 4 min. The second part preparation was prepared by adding CAF to the aqueous dispersion of DPPC and heated on water-bath at 70°C to obtain a lipid solution. Ethanol was added dropwise to the aqueous dispersion of DPPC and CAF under magnetic stirring at 200 rpm and kept aside for 2 h. The rigid sheets of DPPC so formed were washed with phosphate buffer pH 6 to remove the excess ethanol and centrifuged at 2000 rpm. The hydrated ERT dispersion added slowly to DPPC dispersion under magnetic stirring and then kept on water-bath at 46°C (Tm of DPPC) with gentle stirring to form vesosomes.

5.2.4 Method of preparation for hybrid nanoparticles (HNPs)

Self-assembled HNPs for ERT prepared using single-step nanoprecipitation method that involved PLGA and ERT dissolved in acetone under magnetic stirring to form a clear solution. Lipids DPPC and soya lecithin in ratio of 2:1 w/w with the hydrophilic drug CAF dispersed in water and heated at 65–70 °C i.e., above the melting phase transition temperature of lipids to form milky uniform dispersion. The polymer solution was added to lipid dispersion drop-wise under homogenization using lab homogenizer (Remi, India) resulting in polymer nanoprecipitation and formation of lipids self-assembly around the polymer core with hydrophobic interactions. Nanosuspension thus formed was stirred at ambient temperature for 2 h to evaporate the solvents. Lastly 1 mL of PEG-2000 solution added to the nanosuspension with continuous stirring for 30 min followed by probe sonication with 30% amplitude pulse

ON/OFF at 15 sec for 3 cycles. The final nanosuspension was lyophilized in freeze drier (Labconco, USA) for 24 h. [32]

6. CHARACTERIZATION

The optimized nanoparticulate formulations (nanosponges, nanospheres in microneedles, vesosomes and hybrid nanoparticles) were characterized using (Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)), particle size, zeta potential, polydispersity index, solubility studies, % entrapment/ encapsulation efficiency, *in-vitro* release studies, *ex-vivo* release studies, structural chemistry and interactions like Fourier Transform Infrared (FTIR) spectroscopy, Differential scanning calorimeter (DSC), NMR spectroscopy and X-ray diffraction (XRD) spectroscopy, stability studies and animal studies (pharmacokinetics and pharmacodynamics studies, histopathology investigations and serotonin toxicity studies)

6.1 Pharmacokinetics studies

Pharmacokinetics studies were carried out on Sprague-Dawley male rats weighing 180-230 g in compliance with approved protocol CPCSEA/DIPS/150322/25 from the Institutional Animal Ethics Committee (IAEC) in line with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). For the pharmacokinetics studies, the animals were divided into 5, groups (n = 3): plain drug ERT as standard, control (normal saline) and test formulations like nanosponges (NSP3), vesosomes (PD-VS4) and hybrid nanoparticles (HNP-02) administered (20 µL) via the intranasal route. The administered dose for ERT is 1 mg for adults. Animals were sacrificed at selected timepoints (30, 60 and 90 min) by cervical dislocation and brains were carefully dissected out, washed with copious quantity of normal saline, homogenized using polytron homogenizer (Kinematica AG,

Switzerland) and precipitated using methanol as a diluent. Drug content was determined using the validated HPLC method.

6.2 Pharmacodynamics (PD) studies

The pharmacodynamics studies were conducted employing the acetic acid writhing test that induces hyperalgesia, the most significant symptom of migraine in serotonin-controlled animals. Male Wistar rats weighing 220 ± 30 g were selected for study with 5 animals per group. Six groups formed were test sample as nanosponges (NS), microneedles (MN), vesosomes (VS) and hybrid nanoparticles (HNP) with ERT as standard and control. The animals received test samples equivalent to 1 mg of ERT through both nostrils, with normal saline serving as the vehicle. Following an intraperitoneal injection of acetic acid 0.3% w/v, the abdominal constrictions were counted for 15 min to evaluate anti-hyperalgesic activity.

6.3 Histopathology evaluation of brain tissues

The brain tissue was carefully removed after pharmacodynamics studies and immersed in formalin solution for 2h. Haematoxylin and Eosin were used to stain the samples and then examined using an optical microscope (AX80, Olympus, Tokyo, Japan). The tissues were observed for any abnormalities like presence of inflammation, degeneration, necrosis or haemolysis. [33] [34]

6.4 Serotonin toxicity studies

Serotonin toxicity is a potentially life-threatening condition observed due to higher serotonin levels in the body that result in to change in mental status, autonomic hyperactivity, and neuromuscular abnormalities. Overstimulation of post-synaptic 5-HT1A and 5-HT1B receptors leads to an increase in serotonin levels. The male Wistar rats weighing 190-210 g were segregated into six groups (n=5) drug-treated i.e., ERT as standard, formulations like

nanosponges (NS), nanospheres in microneedles (MN), vesosomes (VS) and hybrid nanoparticles (HNP) with nasal saline as control. Post 1 h of intranasal administration of test samples, different neuromuscular excitations were measured as the serotonergic response, and scores were determined based on the presence or absence of behavioural symptoms. Using nominal scale approach for evaluation of serotonin syndrome, the scores were determined based on the presence or absence of response. [35,36]

Statistical analysis

The statistical assessment employed to analysed parameters using Graph pad Prism 5 software (California, USA) with results expressed as mean ± SD. One-way ANOVA followed by Dunnett's multiple comparison test was used to evaluate the data from pharmacodynamics investigations and differences were deemed as statistically significant at (**p 0.001) and highly significant at (***p 0.0001).

7. RESULTS AND DISCUSSION

7.1 Nanosponges

Polymeric, biodegradable and highly porous nanosponges of ERT improved aqueous solubility (50-fold rise), stability (accelerated) and controlled release kinetics (24 h). Based on statistical evaluation significant impact of CAF was seen to improve the solubility of ERT. The solubility rise with cyclodextrin was due to cumulative effect of cyclodextrin-inclusion complex, small particle size and hydrogen bond formation. ERT nanosponges exhibited average particle size of 673.5 ± 11.7 nm and negative zeta potential of -19.4 ± 5.69 mV with controlled *in-vitro* release of 81.6% ± 3.4% for 24 h and *ex-vivo* release of 79% ± 4.2% for 24 h. These nanosponges exhibited higher antioxidant activity 85.68% ±1.23% and better photostability without any effect on nanosponges when exposed to light. FTIR and ^{1}H NMR spectroscopic

studies revealed that ERT and polymeric nanosponges showed significant interaction at indole-derived tetracyclic ring structure of ergoline in cyclodextrin cavity.

7.2 Nanospheres in microneedles (MNs)

The dissolving type of MNs comprised of PLGA nanospheres illustrated particle size in the narrow range of 280.34 ± 6.61 to 320.67 ± 10.86 nm with higher entrapment efficiency of 86.21± 4.52% for ERT. This higher entrapment efficiency was attributed to lipophilic nature of ERT and increased concentration of polymer PLGA along with use of water-immiscible solvent like DCM that increased the solubility. The water-soluble polymer, PVA acted as a stabilizer for primary emulsion prevented particle aggregation and increased % entrapment efficiency. The *in-vitro* release for MNs displayed 52.01 ± 5.71% for ERT and 87.04 ± 2.44% for CAF at 48 h and *ex-vivo* studies illustrated similar results of 51.08 ± 3.56% for ERM and 69.2 ± 2.16% for CFE. The controlled release rate of ERT and CAF-loaded MNs was due to higher viscosity of polymeric matrix of PLGA, PVA and PVP K-90. These hydrophilic polymers form swellable hydrogels that on contact with biofluids cause intentional cracking of the needle tips and enable them to remain in the body for longer time with controlled drug delivery up to 48 h. The FTIR spectroscopy confirmed the entrapment of ERT in swellable polymer matrix of PVA and PVP K-90.

7.3 Vesosomes

The lipid nanoparticles showed average particle size of 315.48 ± 14.27 nm and exhibited good colloidal stability as evident by the zeta potential (-21.78 ± 4.72 mV) along with higher encapsulation efficiency of 91.13 ± 1.29 %. These vesosomes depicted controlled release pattern from *in-vitro* release for ERT (97.65 ± 5.13%) in 48 h and a similar pattern seen in *the ex-vivo* release (82.25 ± 7.47% in 48 h). The impact of enzymatic degradation (PLA_2-induced release profile) on vesosomes and unilamellar vesicles were studied that displayed a burst

release of vesicles in the presence of enzyme, phospholipase A_2, whereas vesosomes demonstrated improved stability and higher protection against enzymatic degradation depicted by the gradual release of contents. TEM analysis showed spherical lipid globules with liposome-in-liposome structure as multivesicular vesicles. The encapsulation of ERT (91.13 ± 1.29 %) in the lipid matrix of vesosomes with enhanced solubility was confirmed by FTIR and ^{1}H NMR spectroscopy. The XRD and DSC findings revealed increased encapsulation of ERT in the lipid vesicles due to conversion of crystalline to amorphous form and creating vesosomes with improved thermal stability.

7.4 Hybrid nanoparticles (HNPs)

The hybrid nanoparticles showed lower particle size (239.46 ± 2.31 nm) and higher entrapment efficiency (86.88 ± 1.67%). Low PDI of 0.196 ± 0.24 (less than 0.3) indicated homogeneity whereas high ZP (-18.36 ± 6.59 mV) demonstrated good colloidal stability attributed to complete coverage of polymer surface by the lipids that inhibit aggregation of particles for prolonged time. Higher entrapment efficiency (86.88 ± 1.67%) was related to lipophilic nature of ERT and its solubilization in lipids such as lecithin and DPPC that formed the shell surrounding the polymer core. The *in-vitro* release studies for HNPs depicted controlled release pattern for ERT (97.12 ± 2.79%) and for CAF (35.26 ± 2.53%) in 48 h and a similar pattern seen in the *ex-vivo* release for ERT and CAF as 75.13 ± 5.62% and 23.03 ± 1.38%, respectively in 48 h. In the presence of biological enzymes and serum, the investigations revealed that HNPs displayed long-term serum stability (24 h) against particle aggregation FTIR spectroscopy confirmed the entrapment of ERT and CAF within self-assembled lipid-polymer matrix by van der Waals forces and hydrophobic interactions. Morphological evaluation by SEM represented homogenous spherical nanoparticles and the multi-layered stacks of lipids around the polymer core depicted the onion-like structure confirmed the lipid coverage around the polymer core.

7.5 Pharmacokinetics studies

Figure 2 illustrated the data of drug concentration in brain for ERT compared with the nanosponges (NS), nanospheres in microneedles (MNs), vesosomes (VS) and hybrid nanoparticles (HNP) at 30,60 and 90 min. The data for ERT showed an increase in brain uptake from 1.25 ng/mL (30 min) to 3.30 ng/mL (60 min) with a decline to the initial level of 1.38 ng/mL at 90 min. The results illustrated that C_{max} for ERT reached within 60 min and then the concentration declined rapidly within 90 min which matched with the prior literature studies of ERT. Moreover, all the formulations illustrated a constant rise in the ERT concentration from 30 to 90 min. The nanosponges depicted concentration of 1.18 ng/mL at 30 min to 2.23 ng/mL at 60 min and 4.47 ng/mL at 90 min. Similar graph was observed for vesosomes with drug concentration gradient from 1.54 ng/mL at 30 min to 1.93 ng/mL at 60 min and 4.69 ng/mL at 90 min. [37] Microneedles showed similar consistent rise in the ERT concentration from 1.34 g/mL at 30 min to 2.26 g/mL at 60 min and 4.84 g/mL at 90 min. However, the formulation HNP showed a steep rise in drug concentration from 1.49 ng/mL at 30 min to 4.75 ng/mL at 60 min and further increased to 5.75 ng/mL till 90 min. Hence, it was concluded that the hybrid nanoparticles exhibited higher absorption in the brain and a further rise in concentration depicted the accumulation in the brain due to the nano-size and entrapment in biodegradable polymer PLGA and the biomimetic lipids.

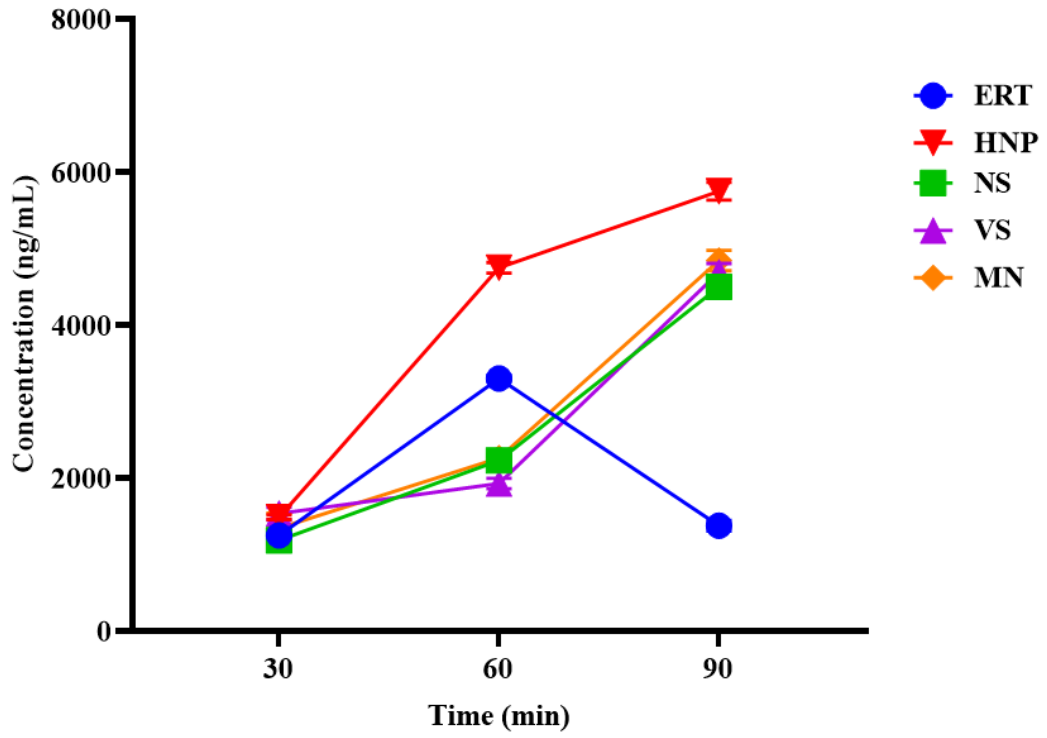

Figure 2. ERT concentration in brain for ERT, HNP, NS, VS and MN

7.6 Pharmacodynamics studies

All the four test samples nanosponges (NS), vesosomes (VS) and hybrid nanoparticles (HNP) along with ERT and control samples were administered intranasally using normal saline as a vehicle and nanospheres in microneedles (MN) were applied with subsequent administration of acetic acid injection intraperitoneally. The number of abdominal constrictions counted for 15 min post-acetic acid injection in all animals (n = 5). Figure 3 illustrated the graphical representation of the data wherein the control sample scored 33.8 whereas ERT-treated group showed 24.6 number of abdominal constrictions. The four formulations depicted the scores of 22.6 for VS-treated group, 23.4 for NS-treated, 24.2 for MN-treated with the lowest value of 17.3 for HNP-treated group.

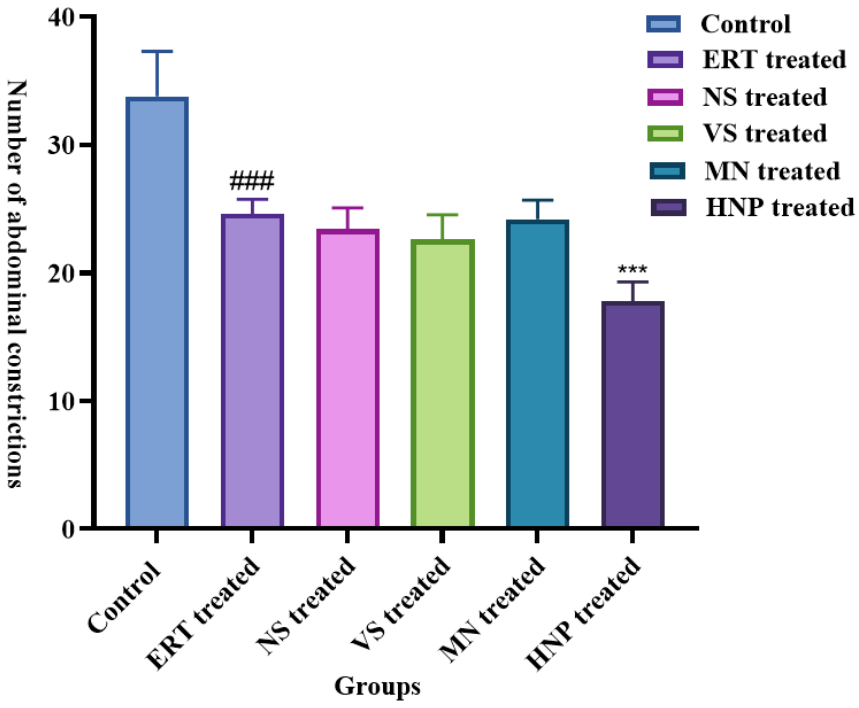

Figure 3. Acetic acid induced writhing test for various groups: Control, ERT-treated, NS-treated, VS-treated, HNP-treated and MN-treated

7.7 Histopathology studies

Figure 4 illustrated the microscopic observations for brain tissue of various groups: control, ERT-treated, NS-treated, VS-treated, HNP-treated and MN-treated groups. The histopathological investigations revealed normal nucleated cells with no abnormalities as compared to control, in all ERT-treated and the four formulation groups. The glial cells were seen of normal size and morphology without signs of inflammation or haemolysis and absence of macrophage infiltration.[38]

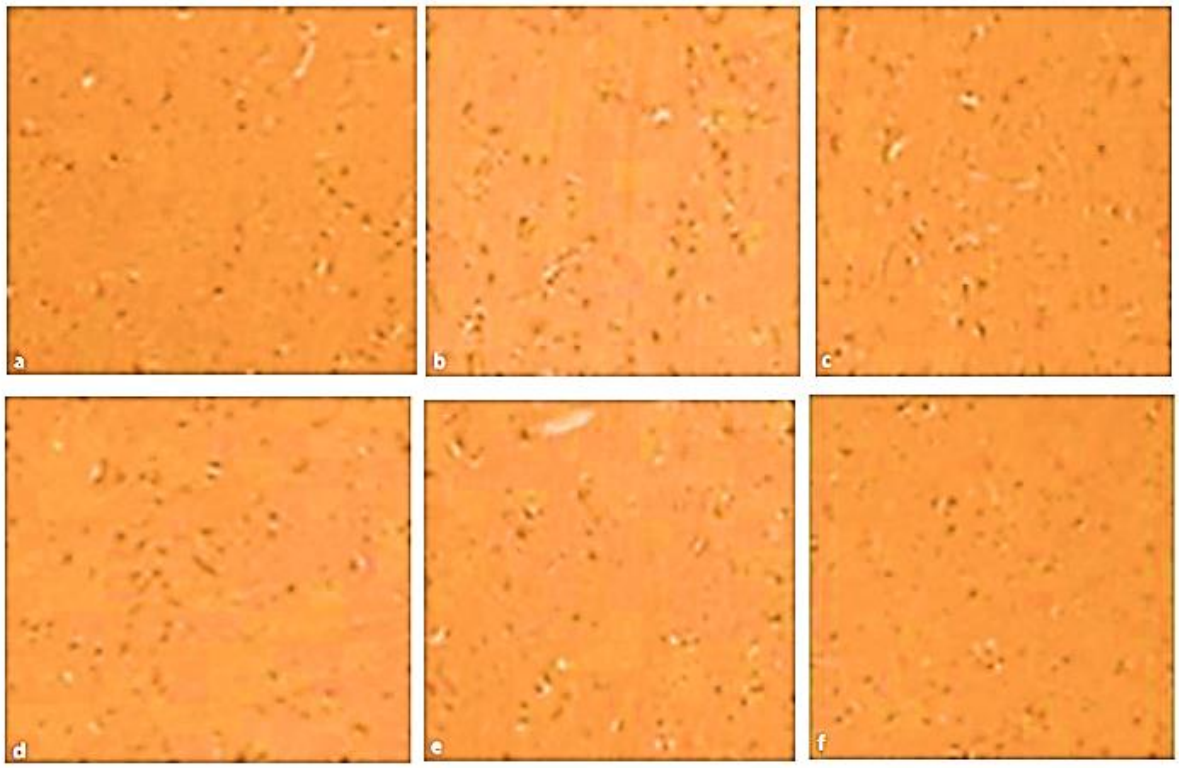

Figure 4 . Histopathological images: a. Control, b. ERT-treated c. NS-treated d. VS-treated e. HNP-treated and f. MN-treated

7.8 Serotonin toxicity studies

The behavioral responses to neuromuscular excitations were assessed using a nominal scale approach viz. categorical response represented as presence or absence of symptoms and the overall scores calculated.

Table 1. Serotonin response score card

Behavioural response 1h post-administration	Untreated	ERT-treated	NS-treated	VS-treated	HNP-treated	MN-treated

Head twitches	0	1	1	1	1	0
Tremors	0	1	0	1	0	0
Hind-limb abduction	0	2	0	0	1	1
Straub tail	0	0	0	0	0	0
Head shaking	0	1	1	1	0	0
Head weaving	0	1	1	1	0	0
Flat low body posture	0	1	1	1	0	0
Backward walking	0	0	1	0	0	0
Hyperactivity	0	2	2	1	2	0
Hyperreactivity	0	2	2	1	2	0
Pilo erection	0	2	1	1	1	0
Defecation	0	1	0	0	0	0

Table 1 revealed the score card wherein the untreated group showed complete absence of responses to the neuromuscular symptoms with score denoted as zero. The ERT-treated group showed a score of 10 out of 12 responses that reported the presence of behavioral responses to various symptoms. The formulation groups illustrated the scores like NS-treated group and VS-treated group scored 8 out of 12 responses with HNP-treated group depicted a score of 5 out of 12 responses whereas MN-treated group showed 1 out of 12 responses, presence for hind limb abduction categorized under neuromuscular excitations. The score denoted based on response received from 1 or 2 animals out of the total 5 animals tested for the presence of response to the symptoms which was termed to be a mild response on the scale of mild-moderate-severe. It was concluded that no severe response was observed for any of the symptoms evaluated by the formulation groups and thus the nanoparticulate formulations showed no serotonin toxicity

and termed to be efficacious in the treatment of migraine. Hence all the four formulations were termed to be safe, non-toxic and efficacious in the treatment of migraine with MNs to be the safest formulation.

Comparative aspects of results of test formulations tabulated in Table 2.

Table 2. Comparison of *in-vivo* parameters for nanosponges, microneedles, vesosomes and hybrid nanoparticles

Parameters	**Polymeric nanoparticles**		**Lipidic nanoparticles**	**Lipid-polymer hybrid nanoparticles**
	Nanosponges	**Nanospheres in microneedles**	**Vesosomes**	**Hybrid nanoparticles**
Formulation development	Ease of scale-up	Complex process	Complex process	Ease of scale-up
Pharmacokinetics	Lowest brain uptake due to strong crosslinking and compact polymer network	Low brain uptake due to strong polymeric matrix	Low brain uptake potential due to multivesicular structure of biomimetic lipids	Highest brain uptake due to core-shell structure of biodegradable polymers and lipids with PEG coating
Concentration in brain (µg/mL)	4.47	4.84	4.69	5.75
Pharmacodynamics (Number of abdominal constrictions)	23.4	24.2	22.6	17.8

Table 2 reveals the comparative data for all four test formulations and based on the results it was concluded that HNP formulation was found to be best among all. The *in-vivo*

characterization such as pharmacokinetic and pharmacodynamic studies demonstrated higher absorption and improved brain uptake potential along with significant reduction in abdominal constrictions that confirmed the anti-hyperalgesic effect of HNP in the effective treatment of migraine.

8. CONCLUSION

The use of nanotechnology coupled with intranasal delivery for all four test formulations resulted in significant improvements in medication delivery with improved stability and wider patient acceptability. For nanosponges the solubility rise with cyclodextrin was due to cumulative effect of cyclodextrin-inclusion complex, small particle size and hydrogen bond formation. The increased extent of crosslinking within cyclodextrin inclusion complex and crosslinker (TDI) during nanosponges formation resulted in a controlled release effect confirmed that nanosponges act as a promising carrier for ERT by improving its solubility and stability. MNs demonstrated to be a rational approach for the efficient transport of ERT via intranasal route using 3D applicator for better management of migraine owing to the painless, effective and safe route for drug delivery. Pharmacodynamic studies revealed the efficacy of fabricated MNs characterized by reduction in number of abdominal constrictions observed post-intraperitoneal injection of acetic acid 0.3% w/v. The development of non-invasive and rapid dissolving MNs comprised of ERT and CAF with usage of 3D applicator device proved to be a viable option in the effective treatment of migraine via intranasal route with reduced long-term risk and wider patient compliance. The lipid nanoparticles were prepared by lipid interdigitation and encapsulation of drugs (ERT and CAF) that resulted in creation of nested bilayer components (vesosomes) for effective treatment of migraine via intranasal route. Vesosomes prevented the leakage of inner unilamellar vesicles by shielding them from the outside environment and enhancing their stability in biological enzymes and serum. Greater vesosomal absorption was observed in the brain during pharmacokinetic studies and higher

intranasal ERT delivery from vesosomes led to a 3.41-fold increase of drug concentration in brain. The PEGylated hybrid nanoparticles were prepared using the nanoprecipitation method with optimum lipid: polymer weight ratio (15%w/w) that exhibited complementary characteristics like higher loading efficiency, biocompatibility and enhanced steric stability. The *in-vivo* results showed greater brain uptake (4.35-fold) with accumulation in brain for longer period of 90 min. These findings verified the protection of ERT by the core-shell structure of HNPs and the outermost lipid-PEG layer prevented the reticuloendothelial uptake. Histopathological examinations revealed absence of haemolysis, inflammation and macrophage infiltration and serotonin toxicity studies exhibited absence of response to various behavioral symptoms that indicated the safe and non-toxic nature of the developed nanoparticulate formulations.

Comparative aspects of results of test formulations tabulated in Table 3.

Table 3. Comparison of parameters for nanosponges, microneedles, vesosomes and hybrid nanoparticles

Parameters	**Polymer nanoparticles**		**Lipid nanoparticles**	**Lipid-polymer hybrid nanoparticles**
	Nanosponges	**Nanospheres in microneedles**	**Vesosomes**	**Hybrid nanoparticles**
Particle size (nm ± S.D.)	693.50 ± 11.7	280.34 ± 6.61	315.48 ± 14.27	239.4 ± 0.28
Zeta Potential (mV ± S.D.)	-19.42 ± 5.69	-20.98 ± 5.12	-21.78 ± 4.72	-18.36 ± 6.59
Polydispersity index (n ± S.D.)	0.19 ± 0.28	0.256 ± 3.21	0.463 ± 0.67	0.196 ± 0.24
Entrapment efficiency %	98.88 ± 2.82	86.21 ± 4.52	91.13 ± 1.29	86.88 ± 1.67

(n ± S.D.)				
In-vitro release % (n ± S.D.)	81.6 ± 3.41	52.01 ± 5.71	97.65 ± 5.13	97.12 ± 2.79
Ex-vivo release % (n ± S.D.)	78.99 ± 4.21	51.08 ± 3.56	82.25 ± 7.47	75.13 ± 5.62

It is concluded that HNP formulation is best among all developed formulations. This HNP formulation involved single step nanoprecipitation method for preparation which can be scaled easily. The characterization results reveal nano-size with satisfactory ZP and PDI as compared to other developed formulations and the core-shell structure offer controlled release profile of 48 h with long-term stability in serum an advantage over liposomes.

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